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TITLE: Development of Methods for the Real-Time and Rapid Identification and Detection of TSE in Living Animals Using Fluorescence Spectroscopy of the Eye

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14. ABSTRACT: Year three of the project, the current reporting period, has shown considerable progress. Our preliminary investigations so far suggest that the most promising part of the eye for revealing spectroscopic signatures of neurological disease is the retina. Our experiments have so far been limited to sheep. Our experiments have been designed to address the following questions: 1. Can ocular spectra be diagnostic of neurological disease? 2. Can the effects of neurological disease be separated from those associated with normal aging? 3. Can images of the eye be obtained that report on neurological disease? Our results indicate that we can answer all three questions in the affirmative.					
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## INTRODUCTION:

Transmissible spongiform encephalopathies (TSEs) are thought to be caused by the accumulation of abnormal protease-resistant proteins called prions, which are found in aging central nervous system tissue and in the eyes. Other protease-resistant compounds, collectively called lipofuscins, also accumulate in CNS. Lipofuscins accumulate in the eye, especially in the diseased eye. An increase in lipofuscin accumulation is known to occur in human Creutzfeldt-Jakob disease victims and in other cases of experimental TSEs. Lipofuscins are fluorescent compounds with characteristic optical spectra. Some individual lipofuscin compounds (especially from the eye) have been studied in detail with regard to optical and chemical properties. The spinal cord and brain also have been observed to be fluorescent under certain wavelengths of light. This is due in part to lipofuscin accumulation in this tissue. The literature indicates that abnormal TSE prions also display characteristic optical spectra. The Principal Investigator's (PI's) preliminary data indicate that the fluorescent spectra of scrapie-infected sheep brain differ substantially from that of the noninfected sheep brain. The purpose of this study is to test the hypothesis that this spectral difference is the result of altered lipofuscin and/or prion spectral properties. Lipofuscins and prions may serve as useful fluorescent markers, which are correlated with the occurrence of TSEs and can be detected by spectroscopy.

## KEY RESEARCH ACCOMPLISHMENTS:

During the first year of this study, we made only marginal progress as a result of difficulties in transmittal of funds to a collaborating laboratory. We dissected sheep and cow eyes and performed fluorescence spectroscopy on all the major eye components and reports that the cornea, lens, retina, and optic nerve show promise. Of these, the optic nerve showed the most potential for changes in spectral properties as a result of prion disease. Unfortunately, because of the lack of control tissues, the only conclusion that can be drawn is that the optic nerve shows the most intense fluorescence. The first year of this project, however, suffered from several setbacks: namely, the inability to transfer funding efficiently to the USDA collaborators and the difficulty of obtaining proper tissue samples. For example, in year one, we were forced to work under the unsatisfactory circumstances of comparing spectra from scrapie-infected sheep eyes with those from healthy cow eyes.

Year two had shown modest improvements in our working conditions. Funds were finally able to be transferred to the USDA collaborators and we were able to establish spectral comparisons between healthy and scrapie infected sheep eyes. The extent of our sampling was not, however, as large as we would like it to be and more importantly, the tissues were not age matched.

Nevertheless, the second year of work had provided the following key results:

- Spectra from the various parts of sheep eyes are very rich in detail as a function of excitation wavelength.
- For purposes of comparison, we presented spectra from three parts of the eye at excitation wavelengths  $\lambda_{ex} = 410, 470, \text{ and } 520 \text{ nm}$ : retina; optic nerve; outer tissue (sclera); and lens.
- Contrary to the conclusions we presented at the end of year one, it appears that while the optic nerve presents the richest spectra with the most detail, the retina and the sclera show the largest differences in spectral features when comparisons are made between the healthy and the infected populations.

Consequently, both tasks 1 and 2 of the Statement of Work, reprinted below continued to be addressed.

Year three of the project, the current reporting period, has shown considerable progress. Our preliminary investigations so far suggest that the most promising part of the eye for revealing spectroscopic signatures of neurological disease is the retina. Our experiments have so far been limited to sheep. Our experiments have been designed to address the following questions:

1. Can ocular spectra be diagnostic of neurological disease?
2. Can the effects of neurological disease be separated from those associated with normal aging?
3. Can images of the eye be obtained that report on neurological disease?

## Statement of Work

### Task 1.

To obtain spectroscopic data from a large statistical sampling of age-matched eyes of healthy and infected animals (mice, hamsters, sheep) in order to verify the hypothesis that TSEs may be detected by fluorescence spectroscopy. Months 1-12:

- a. Obtain statistically significant samples of age-matched healthy and diseased eyes. Because lipofuscin accumulates with age, it is important to distinguish spectroscopic differences arising from age differences from those arising from TSE infection. The limiting step for this Task is the time required “to age” the subjects. All the milestones may be accomplished concurrently. Months 1-12.
- b. Submit the aqueous humor, vitreous humor, lens, retina, and optic nerve to spectroscopic examination by means of steady-state fluorescence and excitation spectroscopy in order to determine whether lipofuscin fluorescence is diagnostic for TSEs. Months 1-12.
- c. In so doing, determine which part of the eye, if any, is more susceptible to yielding information on TSE infection. Months 1-12.
- d. Verify that no other pigments in the eye obfuscate the fluorescent signature arising from the TSEs. Months 1-12.

### Task 2.

To perform an exhaustive study of the fluorescence excitation and emission spectra of solid samples and extracts from diseased and healthy eyes in order to determine the most sensitive and most reliable spectral region to exploit for probing CNS tissue. Months 1-18:

- a. Characterize the fluorescence quantum yield of the lipofuscin pigment extracted from the various parts of the eye. This will require establishing a protocol that successfully removes all the fluorescent pigments from the tissue. The goal is to quantify the number of fluorescent photons that one might expect to detect per 100 incident photons and consequently begin to obtain ideas of the requisite sensitivity of the device that is the ultimate subject of Task 3. In other words, taken as a whole, this information will determine the smallest amount lipofuscin that can be monitored using a given detector and a given excitation wavelength and intensity. Months 1-12.
- b. Compare the fluorescence quantum yield of the isolated pigments with that of the tissue from the eye. Months 12-18.

### Task 3.

To design a prototype device to detect fluorescence from an eye *in vivo*, based upon the spectroscopic evidence accumulated. Months 18-36.

- a. Depending on the results obtained from Tasks 1 and 2, we shall begin with either a green (532-nm) or blue (441-nm) laser source (both available in our laboratories). It is hoped ultimately that laser excitation will not be required because of the expense in the construction of a commercial instrument. We begin with these sources, however, in order to determine the minimum detection threshold that is required to perform a real-time investigation. It is important that the excitation intensities employed not produce damage to the eye of the subject, and these levels shall be carefully monitored. Months 18-24.
- b. These results shall provide sensitivity guidelines. Detection limits will be determined, and possible signals that may interfere will be evaluated. In order to perform a real-time measurement, an optical signal should be detected in 100-300 milliseconds. Months 24-26.
- c. Once these criteria have been met with the best instrumentation available to us (lasers, photomultipliers, CCDs), we shall scale down the technology to provide the most economical solution to the problem. Months 26-36.

## Body

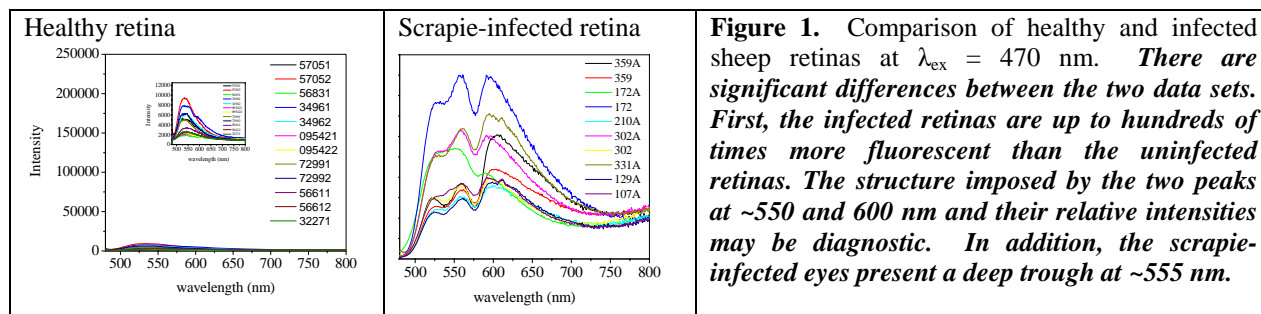
### Key Research Accomplishments

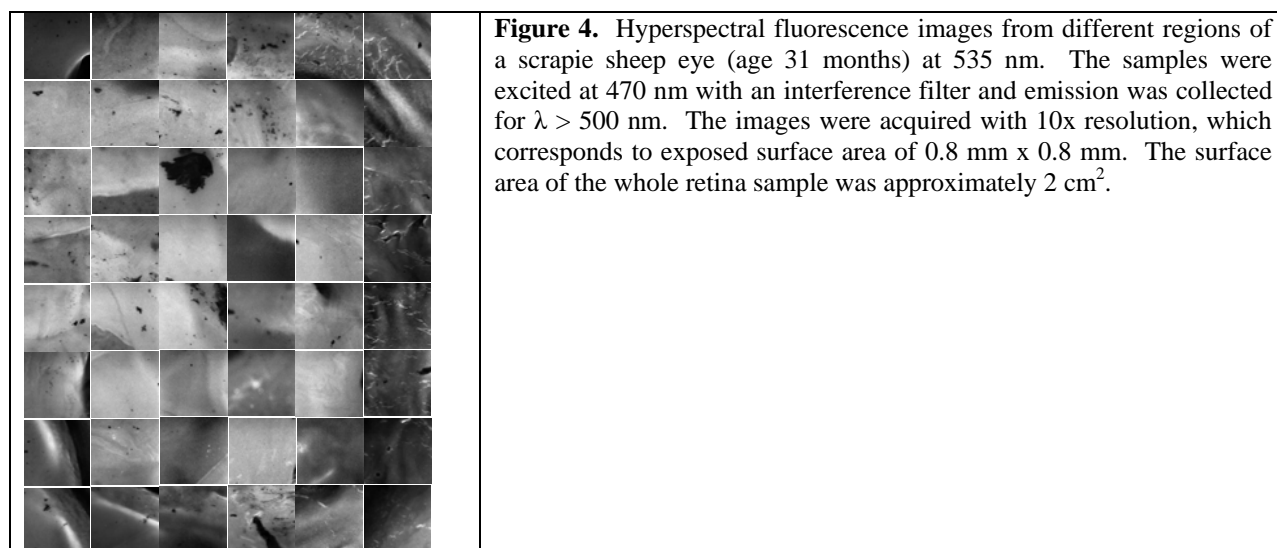
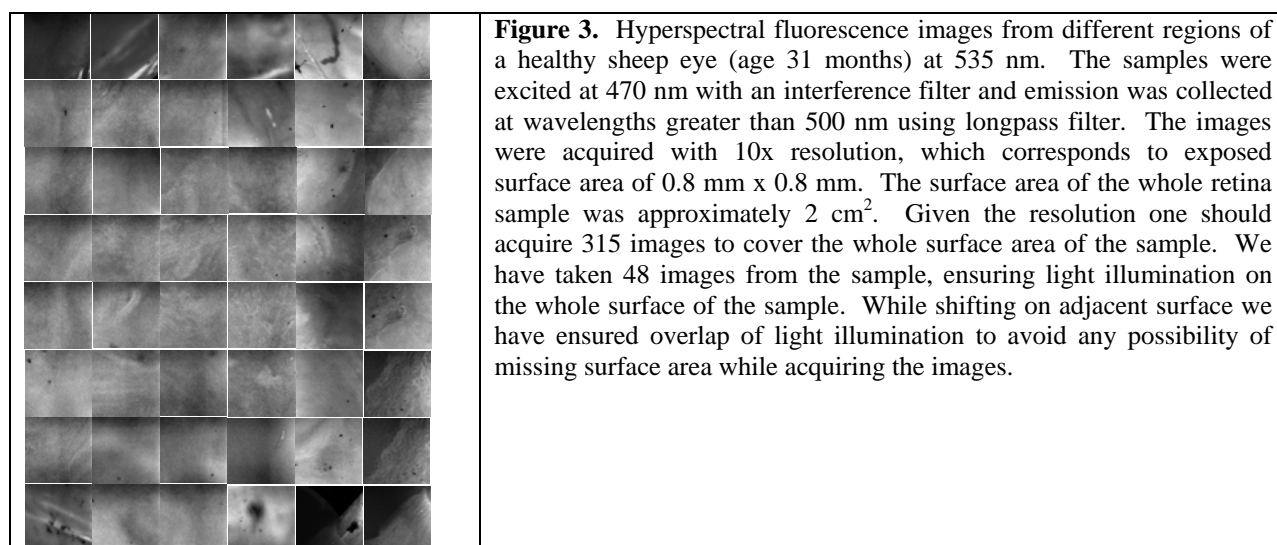
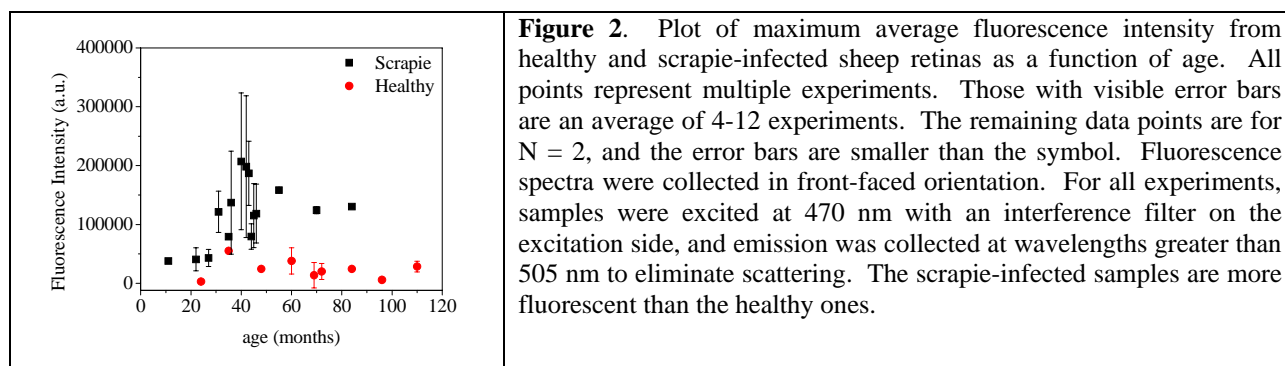
Our results indicate that all three of these questions can be answered affirmatively. First: can ocular spectra be diagnostic of neurological disease? We have been able to show that spectra from the various parts of sheep eyes are very rich in detail as a function of excitation wavelength. The retina and the sclera show the largest differences in spectral features when comparisons are made between the healthy and the infected populations. Fluorescence spectra of healthy and scrapie-infected sheep retina are presented in Figure 1. **The intensity axis is the same for both data sets. The inset for the healthy retinas is a blown-up vertical scale.** The excitation wavelength,  $\lambda_{\text{ex}}$ , is 470 nm. There are significant differences between the two data sets. First, the infected retinas are up to hundreds of times more fluorescent than the uninfected retinas. The structure imposed by the two peaks at ~550 and 600 nm and their relative intensities may be diagnostic. In addition, the scrapie-infected eyes present a deep trough at ~555 nm.

Because of the utility of the structure present in the spectra of the scrapie tissue, particular pains were taken to ensure that these data were not an artifact of the experiment—which involved collecting spectra from solid samples. Although structure was not present in the healthy samples, we needed to ensure that it was nonetheless independent of the polarization of the exciting light, which we have done (data not shown). The structure is in fact the result of a much more heterogeneous environment of pigments giving rise to absorption and emission. We suggest that these are the products of the neurological disease, in this case scrapie. For example, when broad-band (25-nm) excitation is used to excite the sample instead of the narrow-band excitation used in Figure 1, we observe a washing out of the structure.

Second: can the effects of neurological disease be separated from those associated with normal aging? Figure 2 presents a comparison of the maximum fluorescence intensity of the spectra of healthy and scrapie-infected sheep retinas as a function of age. The data indicate that the scrapie-infected retinas are always more fluorescent than the healthy retinas. As can be noted from the Figure, sometimes the error bars are very large. This is a result of the way in which the experiments were performed: namely, exciting the majority of the surface of the retina and collecting light from the entire surface. Such an experiment cannot determine whether the retina is homogeneously weakly emissive or whether it is heterogeneous, containing very bright regions of highly fluorescent material resulting from neurological damage. In order to respond to this concern, we have begun to perform fluorescence microscopy on the retinas.

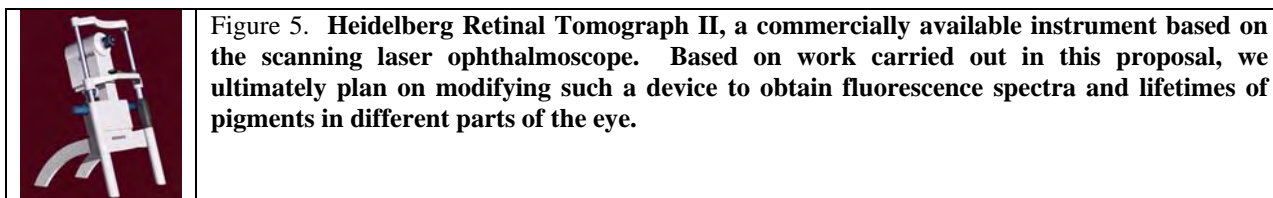
Third: can images of the eye be obtained that report on neurological disease? Figures 3 and 4 present fluorescence microscopy images of age-matched (31-months), nearly entire retinas of healthy and scrapie-infected sheep. While the healthy retina contains fluorescent areas, as expected, these areas lack the structure, brightness, and heterogeneity of the scrapie-infected retina.



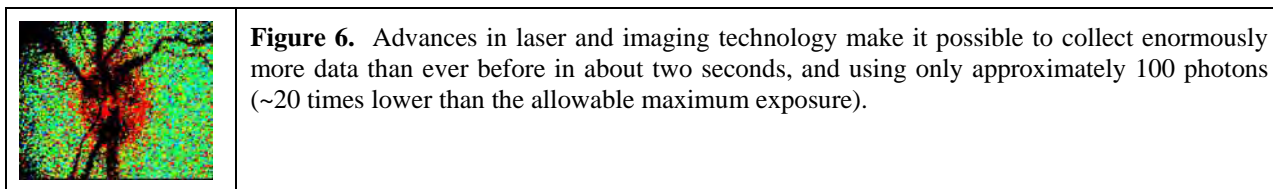


Progress on this project was severely delayed by the process required to obtain authorization to work with TSE-infected materials as well as the difficulty in obtaining materials that were certified as infected or noninfected. Fortunately, we have now found such sources through our collaborators as well as through Dr. Robert G. Rowher of the VA Maryland HealthCare System, who provides scrapie-infected sheep at cost. The data presented here have been collected either with a steady-state fluorometer (Jobin-Yvon, Fluoromax) in our laboratory or with instrumentation in the Carver Microscopy Laboratory. Our data strongly support the idea of using an optical scan to

probe for neurological disease. A possible difficulty, however, may be that these spectral signatures will not be separable from others resulting from ocular disease. Consequently, it will ultimately be necessary to modify a device similar to that depicted in Figure 5 to collect spectra and lifetimes with rapidity and sensitivity. Currently, these devices are only configured to collect images, which severely limits their utility. For example, the device pictured in Figure 5 has recently been configured to image the retina for macular degeneration by measuring the fluorescence lifetime of intrinsic pigments.



Time-resolved methods provide an additional means of discriminating among different pigments that may have the same steady-state spectral characteristics. The laser source for the time-correlated single-photon counting measurements is a home-made, mode-locked Ti-sapphire laser in our laboratory, tunable from 780 to 900 nm with a repetition rate of 82 MHz. This laser is driven by a commercial diode laser, for which we request a service contract to guarantee continual operation. The fundamental from the Ti-sapphire oscillator is frequency doubled by focusing tightly into a 0.4-mm BBO crystal. The resulting blue light, which has a central wavelength of 425 nm, provides the excitation source. The instrument response function of the apparatus has a full-width-at-half-maximum of 80 ps. The eye contains a large number of fluorescing compounds, however, and a static scan cannot discriminate among all of them. Since their instrument allows for time-resolution on a pixel-by-pixel basis, they can also map their fluorescence data onto a photographic image of the structures of the eye, pinpointing the exact anatomical location where a disease process is occurring, for example. Figure 6 depicts a lifetime imaged eye using the modified ophthalmoscope. It is our intent to extend this technology to the lipofuscin type pigments in the eye.



## REPORTABLE OUTCOMES:

For the last funding period, the reportable outcomes were as follows.

- Ms. Tessa Calhoun received her B.S. in chemistry in Spring 2005 and will be entering graduate school in chemistry at the University of California at Berkeley.
- Ms. Erin Campbell received her B.S. in biochemistry in Spring 2005 and will be taking a year off before applying to graduate schools.
- Dr. G. Krishnamoorthy is now Assistant Professor, Department of Chemistry, Indian Institute of Technology, Guwahati, Assam, India.

The outcomes from this period are continuing. We hope to have applied for a patent and have submitted several grant proposals based on the results that we are currently generating.

## CONCLUSIONS:

The major conclusions of the work executed so far are that specific parts of sheep eyes have been identified that may provide spectroscopic signatures of prion disease: these are the retina, lens, and sclera. Surprisingly, the optic nerve did not provide spectroscopic differences between healthy and infected tissue, as was anticipated in the year 1 report. All parts of the eye have been investigated. We note, however, that the samples are not age matched. Given the reproducibility of the spectral features for retina, lens, and sclera, this may prove to be a positive aspect since the

results of Figure 5 demonstrate the increase of autofluorescence from eyes as a function of age. Specific wavelengths have been identified for exciting and detecting useful fluorescence signatures.

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#### APPENDICES:

None.